Neuropharmacology and analgesia

Secoisolariciresinol diglycoside, a flaxseed lignan, exerts analgesic effects in a mouse model of type 1 diabetes: Engagement of antioxidant mechanism

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ABSTRACT

Peripheral painful neuropathy is one of the most common complications in diabetes and necessitates improved treatment. Secoisolariciresinol diglycoside (SDG), a predominant lignan in flaxseed, has been shown in our previous studies to exert antidepressant-like effect. As antidepressant drugs are clinically used to treat chronic neuropathic pain, this work aimed to investigate the potential analgesic efficacy of SDG against diabetic neuropathic pain in a mouse model of type 1 diabetes. We subjected mice to diabetes by a single intraperitoneal (i.p.) injection of streptozotocin (STZ, 200 mg/kg), and Hargreaves test or von Frey test was used to assess thermal hyperalgesia or mechanical allodynia, respectively. Chronic instead of acute SDG treatment (3, 10 or 30 mg/kg, p.o., twice per day for three weeks) ameliorated thermal hyperalgesia and mechanical allodynia in diabetic mice, and these analgesic actions persisted about three days when SDG treatment was terminated. Although chronic treatment of SDG to diabetic mice did not impact on the symptom of hyperglycemia, it greatly attenuated excessive oxidative stress in sciatic nerve and spinal cord tissues, and partially counteracted the condition of weight decrease. Furthermore, the analgesic actions of SDG were abolished by co-treatment with the reactive oxygen species donor tert-butyl hydroperoxide (t-BOOH), but potentiated by the reactive oxygen species scavenger phenyl-N-tert-butylnitrone (PBN). These findings indicate that chronic SDG treatment can correct neuropathic hyperalgesia and allodynia in mice with type 1 diabetes. Mechanistically, the analgesic actions of SDG in diabetic mice may be associated with its antioxidant activity.

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1. Introduction

Diabetic neuropathies are highly prevalent complications in diabetes and cause serious problems afflicting patients, such as chronic neuropathic pain. This painful condition (also known as diabetic neuropathic pain) may occur spontaneously or as a result of exposure to only mildly painful stimuli (hyperalgesia) or to stimuli not normally perceived as noxious (allodynia). Mounting evidence suggests that the management of hyperglycemia alone may be insufficient to prevent or arrest this progressively diabetic complication (Vinik et al., 2000; Martin et al., 2006) and its early identification; on and treatment are of utmost importance for clinicians and diabetic patients (Moore et al., 2009).

Fundamentally different from acute pain treatment that relies on conventional analgesics such as opioids and non-steroidal anti-inflammatory drugs (Eisenberg et al., 2005; Vo et al., 2009), the most effective pharmacological treatment against neuropathic pain is based on the drugs initially developed to treat other CNS...
diseases, i.e. antidepressants and anticonvulsants (Finnerup et al., 2015). Despite being ranked as first-line drugs, these agents (antidepressants and anticonvulsants) could not fully satisfy the clinical need of quenching pain in neuropathic patients, because of modest efficacy, extensive limitations, unwanted side effects and poor patient compliance (Finnerup et al., 2005; 2015). Thus, the development of novel pharmacotherapy to relieve this painful symptom in diabetic patients is in great need.

Flaxseed is a rich source of plant lignans (Thompson et al., 1991). Previous studies suggested the multi-faceted biological activities of flaxseed, such as anti-oxidant and anti-cancer effects (Prasad., 1997; Kitts et al., 1999; Dabrosin et al., 2002), as well as cardiovascular benefits (Prasad., 2009). Secoisolariciresinol diglycoside (SDG) is a predominant lignan in flaxseed. Recently, we revealed that SDG exerted antidepressant-like effect in mice subjected to unpredictable chronic stress (Ma et al., 2013). As antidepressant drugs are clinically used to treat chronic neuropathic pain, we reasoned that SDG may also have therapeutic potential in fighting this persistent painful condition in patients with diabetic neuropathy. Thus, the primary aim of the present study is to investigate the possible antinociceptive capacity of SDG in a mouse model of painful diabetic neuropathy produced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ, 200 mg/kg). Moreover, we evaluated whether chronic SDG treatment can exert beneficial effects on the conditions of hyperglycemia and weight decrease in type 1 diabetic mice, as SDG has also been reported to delay the development of type 2 diabetes in Zucker rat (Prasad., 2001) and improve the indices of glycemic control, insulin resistance and lipid profiles in type 2 diabetic patients (Pan et al., 2007). Finally, after determining SDG antinociception in diabetic mice, we explored its potential action mechanism(s) with focus on its antioxidant potentials (Prasad., 1997; Kitts et al., 1999), since oxidative stress is generally believed as the key pathological process involved in diabetic neuropathy (Vincent et al., 2004; 2005).

2. Materials and methods

2.1. Mice

All experiments were performed using C57BL/6J male mice (7–8 weeks old upon arrival and obtained from the Laboratory Animal Center of Chinese Academy of Sciences). The animals were under good laboratory conditions, housed in groups (4–6 per cage) with food and water available ad libitum and kept in controlled laboratory conditions with the temperature maintained at 22 ± 0.5 °C and a relative humidity of 60 ± 2% in 12 h light cycles (on at 07:00 AM). Experimental behavioral tests were performed in a soundproof and air-regulated room and were done in blind respect to drug treatment. All experiments and animal handling were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ningbo University Committee on Animal Care and Use. The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. Induction of diabetes

Animals (with initial body weight of 21.64 ± 0.52 g) were rendered diabetic by a single intraperitoneal (i.p.) injection of streptozotocin (STZ, purchased from Sigma-Aldrich) at 200 mg/kg. STZ was freshly prepared in citrate buffer (0.1 M, pH 4.5) while age-matched control mice received an equal volume of citrate buffer. The onset of diabetes, following STZ injection, was confirmed by blood glucose levels higher than 250 mg/dl and the blood samples were taken from the tail vein 5 days after the injection of STZ.

Animal body weights were monitored weekly during the experimental period. After 2 weeks of diabetes, only mice that developed evident sensory allodynia (von Frey test) and hyperalgesia (Hargreaves test) were used in further experiments.

2.3. Pharmacological treatments

The treatment with SDG (purchased from Chroma Dex, with a purity of 99.5%) began 20 days after STZ injection when the diabetic mice exhibited significant nociceptive hypersensitivity, i.e. thermal (heat) hyperalgesia and mechanical allodynia. For chronic treatment, the diabetic mice received two oral administrations (morning and evening, as shown in Fig. 1A) of SDG or vehicle per day for 21 consecutive days. For acute treatment, SDG was administered once on day-20 (i.e. 20 days after STZ injection), followed by pain-related behavioral tests at several time points (i.e. 0.5, 1, 2, 3 and 4 h).

Fig. 1. Schematic of secosolariciresinol diglycoside (SDG) administration. (A) Procedures of chronic SDG administration. Twenty days following streptozotocin (STZ, 200 mg/kg) injection, we started chronic treatment with SDG (3, 10 and 30 mg/kg, p.o., twice a day, at 10:00 and 18:00 respectively). During chronic SDG treatment, behavioral testses were done just 1 h before the morning drug administration. After 3 weeks of SDG treatment, tert-butyl hydroperoxide (t-BOOH) and phenyl-N-tert-butyl nitrate (PBN) were co-administered with SDG. (B) Procedures of acute SDG administration. SDG was administered once on day-20 (i.e. 20 days after STZ injection), followed by pain-related behavioral tests at several time points (i.e. 0.5, 1, 2, 3 and 4 h).

After 3 weeks of SDG or vehicle treatment, the mice were co-administered with tert-butyl hydroperoxide (t-BOOH, an reactive oxygen species donor) or phenyl-N-tert-butyl nitrate (PBN, an reactive oxygen species scavenger). The doses of t-BOOH and PBN were selected on the basis of previous study on SDG anti-depression in mice (Ma et al., 2013). For oral administration (p.o., via gavage, with a volume of 10 ml/kg), fresh SDG was dissolved in redistilled water and diluted to the desired concentration on the day of the experiment. After 3 weeks of SDG or vehicle treatment, the mice were co-administered with tert-butyl hydroperoxide (t-BOOH, an reactive oxygen species donor) or phenyl-N-tert-butyl nitrate (PBN, an reactive oxygen species scavenger). The doses of t-BOOH and PBN were selected on the basis of previous studies (Kim et al., 2004; Wang et al., 2010; Yowtak et al., 2011), with minor modification to ensure paucity of intrinsic activity for these antagonists in the present nociceptive tests. For repeated co-administration with SDG, the two agents were i.p. injected twice per day, 30 min before SDG treatment with a volume of 10 ml/kg.
2.4. Nociceptive tests

Thermal hyperalgesia and mechanical allodynia were used as outcome measures of pain-related behaviors and were evaluated by Hargreaves test and von Frey test, respectively. To investigate the acute actions of SDG, the responses to thermal and mechanical stimuli were measured at different time points (e.g. 0.5, 1, 2, 3 and 4 h) after a single SDG administration. In the experimental settings for chronic SDG treatment, the behavior tests were done on day-21, 24, 27, 30, 34, 37, 39 and 41, 1 h before the morning SDG treatment (1st SDG treatment on the day, as shown in Fig. 1B) as reported by previous studies (Benbouzid et al., 2008; Yalcin et al., 2009; Zhao et al., 2012).

Hargreaves test: Thermal (heat) hyperalgesia was assessed based on the Hargreaves procedure (Hargreaves et al., 1988), using a plantar test apparatus (Ugo Basile, Italy) to determine the hindpaw withdrawal latency at a thermal stimulus (radiant heat). Mice were placed in small clear Plexiglas cubicles and allowed to acclimatize for at least 20 min before testing. A radiant heat source with constant intensity (approximately 10 s on average in naïve mice) was focused on the hindpaws of mice and the thermal latency (withdraw latency) was defined as the time (s) from initial heat exposure to withdrawal of the hindpaw. The thermal latency was determined in triplicate for each animal, with 5-min intervals to prevent thermal sensitization and behavioral disturbances. A cut-off time of 22 s was set to prevent tissue damage in the absence of response.

Von Frey test: The mechanical allodynia was measured using a series of von Frey filaments (Stoelting USA) and results (mechanical threshold) were expressed as grams. Mice were placed in transparent acrylic cubicles with an elevated metal grid floor, and allowed to acclimatize for at least 20 min before testing. The mechanical stimulus was applied to the plantar surface of hind paw in a series of ascending forces (equivalent to 0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10 g force, respectively). Each filament was tested five times per paw, and the mechanical threshold was defined as the minimal force that caused at least three withdrawals observed out of five consecutive trials (Zhao et al., 2012).

2.5. Locomotor activity test

The assessment of locomotor activity was performed in mice according to our previous studies (Zhao et al., 2014). Locomotor activity was measured with an ambulometer with five activity chambers (JZ298, Institute of Materia Medica, Chinese Academy of Medical Sciences, China). Mice were placed in the center of the chambers, with their paws connected or disconnected with the active bars producing random configurations that were converted into pulses. The pulses, which are proportional to the locomotor activity of the mice, were automatically recorded for the cumulative total counts of motor activity. Mice were placed in the test chamber 10 min prior to the recording session and then locomotion counts were recorded for 5 min.

2.6. Biochemical tests

Forty-one days after streptozotocin injection, 24 h following the last SDG or vehicle administration, the mice were decapitated for biochemical assessments. The tissues of spinal cord (lumbar L4-L5) and sciatic nerve (proximal to the trifurcation) were quickly dissected out on an ice-chilled glass plate, and were used to measure lipid peroxidation, catalase activity and reduced glutathione (GSH) content.

Lipid peroxidation measurement: In this assay, tissues were immediately cooled in ice and homogenized in 1.15% KCl diluted 1:5 (w/v) containing 1 mM phenyl methyl sulphon fluoride. The homogenates were centrifuged at 1500 g for 20 min and samples of supernatant fluid obtained were frozen at −70°C for further measurements. Protein concentrations of each sample were quantified using bovine serum albumin (BSA) as a standard (Lowry et al., 1951). For the measurement, trichloroacetic acid (10%) was added to the homogenate. This mixture was then centrifuged (5 min, 1000 g). The sample was extracted and thiobarbituric acid (0.67%) was added into the reaction medium. The samples and standards were then placed in a warm water bath (100°C, 20 min). Malondialdehyde, an intermediate product of lipoperoxidation, was quantified by the absorbance at 535 nm and the results are expressed as µmol/mg protein.

Catalase activity assay: The tissues of spinal cord and sciatic nerve were homogenized in 1:10 (w/v) 0.25 M sucrose. The homogenate was centrifuged at 1000 g for 10 min, followed by supernatant being centrifuged at 10,000 g and 4°C for 20 min. Catalase activity was determined by the decrease of absorption at 240 nm in a reaction medium containing 0.05 M phosphate, 1.7 M hydrogen peroxide and 50 µl supernatant fluid with a final volume of 3 ml, and expressed as the amount of hydrogen peroxide decomposed per min per mg of protein (Valsecchi et al., 2008).

GSH measurement: The GSH content was measured spectrophotometrically by its reaction with 5,5′-dithiobis (2-nitro benzoic acid) to yield a yellow colored complex with absorption maximum at 412 nm (Jollow et al., 1974). Shortly, 1.0 ml of post-mitochondrial supernatant (10%) was precipitated with 1.0 ml of sulphydryl reagent (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200 × g for 15 min at 4°C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 mM, pH 7.4) and 0.2 ml 5,5′ dithiobis (2-nitro benzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow color was read at 412 nm and the reduced GSH levels were expressed as µmol/g protein.

2.7. Statistical analysis

All values are presented as the mean ± S.E.M. Data were analyzed by multifactor analysis of variance (ANOVA) or one-way ANOVA. For multifactor-ANOVA, for example, the induction of diabetes (vehicle or STZ injection) and the treatment (vehicle or SDG administration) were done as between-group factors. When needed, the time of measurement (time-course data) was dealt as within-subject factor. The Duncan test was used for post hoc comparisons. For one-way ANOVA, Student–Newman–Keuls test was used for multiple comparisons, followed by Student's test to evaluate the difference between two groups at the same time. Differences with P < 0.05 were considered statistically significant.

3. Results

3.1. Effects of SDG on animal body weight, blood glucose and glycosylated hemoglobin (HbALc) in control (non-diabetic) and diabetic mice

As shown in Table 1, compared with that of control mice, the body weight of type 1 diabetic mice decreased while the levels of blood glucose and HbA1c increased over the 40-day experimental period, although at baseline level there were no significant differences between grouped mice in all these profiles. Chronic SDG treatment increased the body weight of diabetic mice toward normal level and was greater than that of vehicle-treated diabetic mice (F3,39 = 6.3, P < 0.01). However, daily administration of SDG for three weeks did not exert any beneficial effect on hyperglycaemic condition and escalated level of HbA1c in diabetic mice.
Table 1
Effect of SDG on levels of body weight, blood glucose and HbA1c in control and diabetic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control mice</td>
<td>Diabetic mice</td>
<td>Control mice</td>
</tr>
<tr>
<td>Vehicle</td>
<td>28.52 ± 0.68</td>
<td>21.16 ± 0.67</td>
<td>116.82 ± 7.88</td>
</tr>
<tr>
<td>SDG-3 mg/kg</td>
<td>21.73 ± 0.72</td>
<td>332.49 ± 31.03</td>
<td>11.16 ± 0.71</td>
</tr>
<tr>
<td>SDG-10 mg/kg</td>
<td>22.30 ± 0.55</td>
<td>352.15 ± 38.94</td>
<td></td>
</tr>
<tr>
<td>SDG-30 mg/kg</td>
<td>28.35 ± 0.59</td>
<td>24.93 ± 0.61</td>
<td>118.02 ± 12.55</td>
</tr>
</tbody>
</table>

*: not evaluated. Values were expressed as mean values ± SEM of 9–12 mice.

\( * P < 0.01 \), compared with vehicle treated control mice.

\( * P < 0.01 \), compared with vehicle treated diabetic mice.

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**Fig. 2.** Effects of chronic secoisolariciresinol diglycoside (SDG) treatment on mechanical allodynia and thermal hyperalgesia in diabetic mice. The SDG treatment started 20 days following streptozotocin (STZ, 200 mg/kg) injection. (A) Effect of chronic treatment with SDG on the thermal sensitivity (Hargreaves test) in control and diabetic mice. Chronic SDG treatment (30 mg/kg, p.o., twice per day for three weeks) corrected thermal hyperalgesia in diabetic mice (left panel for time-course) and this antihyperalgesic action was dose-dependent (3, 10 and 30 mg/kg, right panel), without affecting the measures in control (non-diabetic) mice. (B) Effect of chronic treatment with SDG on the mechanical sensitivity (von Frey test) in control and neuropathic mice. Chronic SDG treatment (30 mg/kg, p.o., twice per day for three weeks) normalized mechanical allodynia in neuropathic mice (left panel for time-course) and this antiallodynia was dose-dependent (3, 10 and 30 mg/kg, right panel), without affecting the measures in control mice. Data are expressed as mean ± S.E.M (n=9–12 per group), evaluated by multifactor ANOVA followed by Duncan test and one-way ANOVA followed by Student–Newman–Keuls test.
3.2. Chronic but not acute SDG treatment ameliorated thermal hyperalgesia and mechanical allodynia in mice with diabetic neuropathy

The neuropathy induced by STZ-injection produced in mice neuropathic thermal hyperalgesia and mechanical allodynia, which were persistent (lasting for at least 8 weeks) and maintained throughout the experiment (Fig. 2A and B). In contrast, the nocifensive sensitivity to thermal or mechanical stimuli was not altered in control (non-diabetic) mice.

After 3 weeks treatment, SDG markedly attenuated in diabetic mice the pronounced thermal hyperalgesia ($F_{7,286} = 10.6, P < 0.01$; Fig. 2A, left panel) and mechanical allodynia ($F_{7,291} = 14.3, P < 0.01$; Fig. 2B, left panel) in a dose-dependent manner ($F_{3,40} = 24.7, P < 0.01$ for Hargreaves test, post hoc: control mice = diabetic mice + SDG 30 mg/kg > diabetic mice + SDG 10 mg/kg > diabetic mice + SDG 3 mg/kg = diabetic mice + vehicle, Fig. 2A, right panel; $F_{3,41} = 55.2, P < 0.01$ for von-Frey test, post hoc: control mice = diabetic mice + SDG 30 mg/kg > diabetic mice + SDG 10 mg/kg > diabetic mice + SDG 3 mg/kg = diabetic mice + vehicle, Fig. 2B, right panel). At the highest dose of 30 mg/kg, SDG showed recuperating effects on the thermal hyperalgesia and mechanical allodynia in diabetic mice, without influencing the measures in control (non-diabetic) mice (Fig. 2A and B).

We also evaluated the effects of acute SDG administration on the nocifensive behaviors in control (non-diabetic) and diabetic mice. As shown in Fig. 3A and B, acute SDG treatment (3, 10 and 30 mg/kg, p.o.) did not influence the nociceptive sensitivity in Hargreaves test and von Frey test regardless of control and diabetic mice.

To determine the possible effect of SDG on motor response, we performed additional experiments to investigate the effects of acute and chronic SDG treatment on locomotor activity in control and diabetic mice. As shown in supporting information (Fig. S1), neither acute nor chronic SDG treatment influenced locomotor activity in both control and diabetic mice, implying its paucity of locomotion and motor interfering.

3.3. Effect of SDG withdrawal following three weeks of treatment on the pain-related behaviors in mice

As shown in Fig. 4, we terminated SDG treatment on day 36

![Graphs showing the effects of SDG treatment on pain-related behaviors in mice](image)}

Fig. 3. Effect of acute secoisolariciresinol diglycoside (SDG) administration on thermal latency and mechanical threshold in control and diabetic mice. Acute SDG administration started 20 days following STZ injection. After baseline (0 h) assay of thermal (A, Hargreaves test) and mechanical (B, von Frey test) sensitivity, mice (control or diabetic) were administered with SDG (30 mg/kg, p.o.) and then tested at 0.5, 1, 2, 3 and 4 h following SDG administration. (A) Acute SDG treatment did not impact on the thermal sensitivity in both control and diabetic mice. (B) There is no alteration in mechanical sensitivity after single treatment with SDG. Data are expressed as mean ± S.E. M (n=9–12 per group), assessed by multifactor ANOVA followed by Duncan test.
ANOVA followed by Duncan test. peroxidation (Fig. 5A), and depressed catalase activity (Fig. 5B) and nerve and spinal cord, as evidenced by escalated levels of lipid peroxidation (Fig. 5A), and increased levels of catalase activity (Fig. 5B) and GSH (Fig. 5C) in tissues of sciatic nerve and spinal cord, compared with those in vehicle-treated diabetic mice.

3.5. SDG antinociception was abolished by co-treatment with a reactive oxygen species donor tert-butyl hydroperoxide (t-BOOH), but potentiated by a reactive oxygen species scavenger phenyl-N-tert-butyl nitroso (PBN).

To investigate whether the antioxidant mechanism is specifically required for the antihyperalgesic and antiallodynic effects of SDG, we investigated whether the two effects were influenced by co-administration of the reactive oxygen species donor t-BOOH or the reactive oxygen species scavenger PBN. After 3 weeks of vehicle or SDG treatment, diabetic or control mice were co-administered with t-BOOH or PBN. As shown in Fig. 6, the antinociceptive effects of SDG in diabetic mice were abrogated by repeated co-administration of t-BOOH (0.2 mg/kg) (Hargreaves test: \( F_{6,227} = 9.7, P < 0.01 \), Fig. 6A, left panel; von-Frey test: \( F_{6,229} = 15.6, P < 0.01 \), Fig. 6B, left panel), even though the first co-administration had no acute inhibiting effect (data not shown). However, t-BOOH did not impact on the nociceptive sensitivity to thermal or mechanical stimuli in vehicle-treated diabetic mice and age-matched control (non-diabetic) mice, indicating t-BOOH specifically counteracted SDG analgesia in diabetic mice. In PBN co-administration test, we investigated whether SDG analgesia could be altered by PBN. As shown in Fig. 6, administration of PBN (10 mg/kg) or ineffective dose of SDG (3 mg/kg) alone did not affect the nociceptive sensitivity in Hargreaves test (Fig. 6A, right panel) and von Frey test (Fig. 6B, right panel) for both control and diabetic mice. Nevertheless, co-administration of them engendered robust antinociceptive effects in diabetic mice (Hargreaves test: \( F_{6,224} = 8.7, P < 0.01 \), Fig. 6A, right panel; von-Frey test: \( F_{6,228} = 11.0, P < 0.01 \), Fig. 6B, right panel), suggestive of a potentiating effect when SDG was co-treated with PBN. In light of these results, it should be presumed that the antioxidant mechanism may be causally responsible for the antihyperalgesic and antiallodynic effects of SDG.

4. Discussion

In the present study, we show that secoisolariciresinol diglycoside (SDG), when dosed chronically and orally in mice with painful diabetic neuropathy, exerted antihyperalgesic and antiallodynic effects, although it did not improve the symptoms of hyperglycemia and only partially attenuated the condition of weight decrease. Mechanistically, the antinociceptive effects of SDG may be related to its antioxidant property, since it corrected the overproduction of lipid peroxidation in sciatic nerve and spinal cord of diabetic mice and its analgesic actions were modified by pharmacologically altering in vivo redox level.

Diabetes is one of the leading causes of painful peripheral neuropathy, which is featured by damage to peripheral nerves causing peripheral or central hypersensitivity (a process of sensitization). To date, although a strict glycemic control has been proven to be effective to prevent progression of neuropathy and reduce the risk of developing diabetic neuropathic pain (Vinik et al., 2000; Martin et al., 2006), a treatment with a favorable risk/benefit profile against established pain is lacking. Current pharmacotherapy to alleviate diabetic neuropathic pain hinges heavily on the same serial of antidepressants and anticonvulsants used as first-line drugs treating chronic neuropathic pain (Ziegler, 2008). However, all of these drugs provide relief in only a sub-fraction of patients and treat the symptoms but not the causes, with...
Fig. 5. Effect of secoisolariciresinol diglycoside (SDG) treatment (3, 10 and 30 mg/kg, p.o., twice per day for three weeks) on profiles of lipid peroxidation, catalase activity and reduced GSH content in mice tissues of sciatic nerve and spinal cord. Compared with control mice, diabetic mice showed increased levels of lipid peroxidation (A), and decreased level of catalase activity (B) and GSH content (C) in tissues of sciatic nerve (left panels in A, B and C) and spinal cord (right panels in A, B and C). Following three weeks of treatment, SDG ameliorated, in a dose-dependent manner, these aberrant levels of lipid peroxidation (A), catalase activity (B) and GSH content (C) in tissues of sciatic nerve (left panels) and spinal cord (right panels), without influencing the measures in control mice. Data are expressed as mean ± S.E.M (n=9–12 per group), evaluated by two-way ANOVA followed by Duncan test.
significant side effects that limit their use. Secoisolariciresinol diglycoside (SDG) is a predominant lignan in flaxseed and has been shown to delay the development of type 2 diabetes in Zucker rat (Prasad, 2001). Furthermore, we recently found that SDG exhibited antidepressant-like activity in mice (Ma et al., 2013). These findings motivated us to investigate the potential analgesic efficacy of SDG against painful diabetic neuropathy. We injected STZ to mice to produce the mouse model of diabetes since the STZ-induced diabetes is the most widely used experimental model mimicking human diabetes (Rees and Alcolado, 2005). In the present study, STZ-treated mice displayed early signs of sensory defects (i.e. thermal hyperalgesia and mechanical allodynia), similar to what observed in the human disease.

The main finding of the present study is the ability of SDG, following three weeks of oral administration, to induce relief of neuropathic hyperalgesia and allodynia in mice with type 1 diabetes. To our best knowledge, it is the first report to demonstrate the antinociceptive activity of SDG and its analgesic action incorporates several characteristic aspects. Firstly, the anti-hyperalgesia and antiallodynia by SDG are reversing actions (not a prophylactic one) as the treatment started on the 20th day following STZ injection when the pain-related behaviors had been established, thus implying its potential utility in the development of novel and clinically relevant drugs for the treatment of painful

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**Fig. 6.** Effects of co-administration of the reactive oxygen species donor tert-buty1 hydroperoxide (t-BOOH) and the reactive oxygen species scavenger phenyl-N-tert-butyl nitroxine (PBN) on the analgesic effects of secoisolariciresinol diglycoside (SDG). The analgesic actions of SDG in diabetic mice were abolished by repeated co-administration of t-BOOH (0.2 mg/kg, i.p.) in both Hargreaves test (A, left panel) and von-Frey test (B, left panel). A singular administration of PBN (10 mg/kg, i.p.) or SDG at ineffective dose (3 mg/kg, p.o.) did not exhibit any analgesic effect in the Hargreaves test (A, right panel) and von-Frey test (B, right panel) regardless of control or diabetic mice, but co-administration of them engendered remarkable antihyperalgesic (A, right panel) and antiallodynic (B, right panel) effects in diabetic (not control) mice. Data are expressed as mean ± S.E.M (n = 9–12 per group), assessed by multifactor ANOVA followed by Duncan test.
diabetic neuropathy. Accordingly, it would be intriguing to know whether SDG can prevent the development of chronic pain by a preemptive administration. Second, its analgesic actions are time dependent, inasmuch as they were observed following repetitive (about 10 days) but not a single dose SDG regimen. Likewise, it was shown that the analgesic actions of SDG were remained for about three days after the termination of SDG treatment. Third, the antinociception by SDG shown in the present study should be a genuine one, since the pain-related behavioral tests were performed on the next morning following the last SDG administration and thus the antinociceptive actions are persistent for at least one day. This assertion is strengthened by another fact that the analgesic actions of SDG persisted for three days following SDG treatment was terminated. Thus, it is presumed that the SDG analgesia against painful diabetic neuropathy is a persistent action rather than a transient one. Lastly, the analgesic actions of SDG may be not ascribed to the control of hyperglycemia. Different from previous studies reporting the beneficial actions of SDG on diabetic animals (Prasad, 2001; Moree et al., 2013), we did not observe any effect of SDG on the hyperglycemia in type 1 diabetic mice. This discrepancy concerning SDG action on symptom of hyperglycemia in diabetic animals may correlate with some factors such as types of diabetes (type 1 versus type 2), animal species (mice versus rats) and STZ doses (200 mg/kg versus 60 mg/kg). Therefore, SDG may not have favorable effect on the control of hyperglycemia in type 1 diabetic mice as it did in the cases of type 1 and 2 diabetic rats. Accordingly, the partial recovery of weight decrease in diabetic mice should be also unrelated to the improvement of hyperglycemia by SDG, but may be the consequence of pain relief.

An important sequelae of chronic hyperglycemia is the enhanced oxidative stress resulting from an imbalance between production and neutralization of reactive oxygen species. Reactive oxygen species are pro-oxidant factors in diabetes and lipid peroxide-mediated tissue damage has been observed in type 1 and type 2 diabetes (Feillet-Coudray et al., 1999). The antioxidant enzyme defense system is also attenuated in the peripheral nerves of diabetic animals indicating the critical role of oxidative stress in diabetic neuropathy (Low et al., 1997). Accordingly, some compounds with antioxidant properties have been reported to exert beneficial effects on diabetic neuropathic pain (Comelli et al., 2010; Valsecchi et al., 2011; Zhang et al., 2013). Given that SDG has been shown to possess antioxidant activity (Prasad, 1997; Kitts et al., 1999), we reasoned that its analgesia against painful diabetic neuropathy shown in the present study may correlate with its antioxidant bioactivity. This mechanistic assumption was corroborated by experimental evidence demonstrated here. First, we characterized the profiles of oxidative stress by measuring the lipid peroxidation with the thiobarbituric acid reactive substances assay. Consistent with previous reports (Valsecchi et al., 2011; Zhang et al., 2013), hyperglycemia induced an escalation of lipid peroxide levels in tissues of spinal cord and sciatic nerve when compared with that of non-diabetic control. Following chronic SDG treatment, these aberrant lipid profiles were significantly corrected in a dose-dependent manner, with a reversing effect at the highest SDG dose (30 mg/kg). These results suggest that chronic SDG administration can normalize the condition of oxidative stress in diabetic mice. But a mechanistic concern is raised as whether the correction of the overproduction of lipid peroxidation is causally correlated with the therapeutic actions of SDG against diabetic neuropathic pain or this outcome is only a sequela of pain palliation. To address this question, we investigated whether the analgesic actions of SDG can be influenced by co-administration of the reactive oxygen species donor tert-butyl hydroperoxide (t-BOOH) or the reactive oxygen species scavenger phenyl-N-tert-butylnitrone (PBN). Our results clearly show the antihyperalgesia and antiallodynia exerted by SDG were abrogated by co-treatment with t-BOOH while potentiated by PBN. These results provide pharmacological evidence supporting the antioxidant mechanism underlying SDG analgesia in the context of painful diabetic neuropathy. It should be pointed out here that the doses of t-BOOH (1 mg/kg, i.p.) and PBN (10 mg/kg, i.p.) used in this study are relative low and thus paucity of intrinsic activity in the present pain-related behavioral tests, i.e. t-BOOH at the dose of 1 mg/kg or PBN at the dose of 10 mg/kg per se did not alter the sensory sensitivities in control mice or vehicle-treated diabetic mice. In contrast, higher doses of t-BOOH (10 mg/kg, i.p.) and PBN (100 mg/kg, i.p.) have been reported to impact on nociceptive behaviors in animal models, with t-BOOH being pro-nociceptive (Gwak et al., 2013) while PBN being anti-nociceptive (Kim et al., 2010).

In conclusion, the present study demonstrates that daily treatment with SDG for three weeks in mice with type 1 diabetes can provide dose-dependent alleviation of neuropathic thermal (to heat) and mechanical hypersensitivity. Although SDG did not impact on the symptom of hyperglycemia in diabetic mice, it greatly attenuated the overproduction of lipid peroxidation in tissues of sciatic nerve and spinal cord. Furthermore, SDG analgesia was abolished by the reactive oxygen species donor t-BOOH, but potentiated by the reactive oxygen species scavenger PBN. It is thus convincing that the antioxidant mechanism may underlie the analgesic actions of SDG.

Acknowledgments

This project was sponsored by National Basic Research Program of China (2015CB553504), National Natural Science Foundation of Ningbo (2012A610249, 2013A610248, 2013A610254, 2013A610257) and China (81202814, 81471350, U1132602), Ningbo Key Laboratory of Sleep Medicine (2012AZ2001), Shanghai Municipal Commission of Health and Family Planning (20124Y116), Changing Committee of Science and Technology in Shanghai (CNKW2013F04).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.10.024.

References


